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Mode of Action of Antifilarials: Modulation of Immune Adherence to Microfilariae in vitro

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Abstract: Enhancement of in vitro cellular adherence to microfilariae (mf) has been regarded as an important phenomenon in microfilaricidal action of diethylcarbamazine. Modulation of in vitro cell-adherence to sheathed (Brugia malayi and Litomosoides carinii) and unsheathed (Acanthocheilonema viteae) mf by various antifilarials has been investigated using splenocytes from naive mastomys and cotton rats (Sigmodon hispidus). All the piperazine derivatives (diethylcarbamazine, Centperazine, N-oxide a major metabolite of DEC) enhanced antibody-mediated cell-adherence to both sheathed and unsheathed mf in vitro but not levamisole, a compound with a different chemical structure. Among all piperazines, maximum promotion of cell-adherence was exerted by N-oxide followed by Centperazine (a piperazine compound synthesised in our Institute) and DEC in that order. Sheathed mf of B. malayi and L. carinii required complement and specific IgG antibody (from rabbit immunized with specific antigens) while cell-adhesion was complement independent in case of unsheathed mf of A. viteae requiring specific IgM antibody (from infected mastomys). Maximum adherence occurred in presence of 90–120 days old infected serum whether microfilariae were isolated from blood or uteri of female worm, however, adhesion was comparatively very low in latter case. Intensity of cells adhered was proportional to percent mf showing cell-adherence. Cell adherence decreased with increasing dilution of antibody.

Key words: Acanthocheilonema viteae, Brugia malayi, Litomosoides carinii, Mastomys natalensis, Antifilarials, Diethylcarbamazine, Centperazine, DEC N-oxide, Levamisole, Cell-adherence, Microfilariae, Complement, IgG, IgM

INTRODUCTION

Diethylcarbamazine (DEC) is the drug of choice against filariasis for four decades, however, its mode of action is not yet clearly understood. According to the findings of Piessens and Beldekas (1979), Subrahmanyam (1983), DEC promotes antibody dependent cellular adherence to microfilariae. Experiments were therefore designed to see whether this adhesion promoting action is an exclusive property of DEC or extended to other active piperazine analogues like centperazine (Saxena et al., 1978), N-oxide, a major
metabolite of DEC and also an agent with a different chemical entity like levamisole. Both sheathed (*Litomosoides carinii, Brugia malayi*) and unsheathed (*Acanthocheilonema viteae*) mf were selected in the study in order to observe whether these two sorts of mf respond to different antifilarials in a similar manner or not.

**MATERIALS AND METHODS**

**Infection:**

Male *Mastomys natalensis* (6 wk old) were infected with *A. viteae* (Singh et al., 1985) by subcutaneous inoculation of 50 infective larvae (*L*₂) each recovered from previously exposed ticks (*Ornithodoros moubata*). 6 wk old male mastomys were infected with *B. malayi* (Murthy et al., 1983) by subcutaneous inoculation of 100 *L*₂ recovered from previously infected mosquitoes (*Aedes aegypti*) fed on microfilaraemic *M. natalensis* 10–11 days back. 6 wk old cotton rats of male sex were also infected by exposing them to previously infected mites (*Liponyssus bacoti*) (Misra et al., 1983).

**Immunisation of rabbits:**

Three male (1½ kg) rabbits were immunised each with homogenates of Adult *A. viteae, B. malayi* and *L. carinii*. Immunisation consisted of 3 doses of 500 µg antigen protein per rabbit on days 0, 15 and 30, first dose was administered along with Freund’s complete adjuvant. Booster doses of antigens were administered until rabbits developed high antibody titres.

**Microfilariae:**

Mf were isolated from the blood of infected rodents using millipore filter (Singh et al., 1985). *B. malayi* and *A. viteae* mf were isolated with 5.0 µ pore size filter while for *L. carinii* 3 µ filter was used. Blood for *A. viteae* infected mastomys was taken after anaesthetising the animals with Nembutal (40 mg/kg). Microfilariae of *A. viteae* were also collected after incubating the adult females *in vitro* at 37°C in medium RPMI 1640 with penicillin (100 units/ml) and streptomycin sulphate (100 µg/ml).

**Cells:**

Splenocytes for cell-adherence assay were recovered from the spleen of infected and normal mastomys and cotton rats. Briefly, tissues were teased in the medium and passed through a sieve. Cells were then washed and suspended in the medium. Finally their viability was assessed by Trypan blue exclusion test.

**Sera samples**

Sera were collected from the blood of infected mastomys and cotton rats at different durations of infection (15, 45, 90, 120 & 200 day). Sera were also isolated from the blood of normal mastomys, cotton rats and rabbits under identical conditions. Blood was also drawn from the ear vein of rabbits immunised with 3 different antigens and sera were separated.

**Immunoglobulins (Igs)**

IgM (19S) and IgG (7S) were separated from infected and immune sera by Sephadex G-200 column followed by purification with DEAE-cellulose (Tanner and Weiss, 1978).
Separated immunoglobulins as well as immune and normal sera samples were then used in cell-adherence assay.

**Heat treatment of sera**

In order to observe the role of complement and thermo-labile antibody (IgE) in mediating cellular adherence to mf in vitro, sera samples were heated at 56°C for 30 min and 2 hrs respectively in a water bath.

**Adherence assay**

Assay was carried out in microtitre plates. Approximately 200 living mf and $10^6$ viable cells were added to each well containing Medium RPMI 1640 supplemented with serum or purified immunoglobulin. Final volume in each well was 150 µl which contained 50 µl of serum or Ig, 50 µl splenocytes ($10^9$), 25 µl mf (200) and 25 µl medium. Plates were kept at 37°C in a humid cabinet. Cell-adherence and motility of microfilariae was observed after 1, 3, 6 and 24 hrs of incubation. Percent adherence (% mf with 5 or more cells adhered) as well as intensity of cells adhered to single mf was assessed by microscopic examination of 100 mf in each well.

**Factors affecting cell-adherence:**

**Duration of Infection**

Sera samples from infected cotton rats and mastomys were collected on days 15, 45, 90, 120 and 200 post infection to see their relative adherence capacity to mf at different durations of infection.

**Blood and uterine mf**

Microfilariae of *A. viteae* isolated from blood and those released by adult females in vitro into the medium were used under identical conditions in order to investigate the in vitro adherence phenomenon with these two sorts of microfilariae.

**Sera dilution**

Immune sera were serially two fold diluted in order to evaluate the degree of cell-adherence to three species of mf with increasing dilution of antibody. Cell-adherence was considered positive when mf had 5 or more adhered cells.

**Assay of adherence reaction in presence of antifilarials**

The general procedures of adherence assay in presence of drug were the same as mentioned earlier. However, to incorporate maximum efficiency in experimental conditions, different ingredients to be put into the wells of microtitre plate were chosen in such a manner so that around 50 per cent of the microfilariae were adhered with cells. Results obtained from our experiments on factors affecting cell-adherence assay were taken into consideration for the purpose. Thus for microfilariae of *L. carinii*, immune rabbit serum was used whereas for microfilariae of *A. viteae*, 120 days old homologous infected mastomys serum was applied and for *B. malayi* microfilariae, serum of rabbit immunised with homologous adult *B. malayi* antigens were used. As undiluted serum usually showed high percentage of cellular adherence, diluted serum at 1:8 was used to have around 50% cell-adherence. Monitoring of results at different timings indicated 3 hours to be the optimal time of incubation to achieve maximum amount of cell-adherence reaction. Diethylcarbamazine (Cynamide, India), Centperazine and N-oxide of DEC (synthesised at C. D. R. I.,
Lucknow) and levamisole (Unichem, Bombay) were used in the assay. Individual drug was used at 2-fold dilutions starting from 1 mg/ml concentration.

RESULTS

Maximum cellular adherence (91.5%) occurred in mf of *A. viteae* when infected mastomys serum (120 d old) was used, however, no cell-adherence was observed with serum of rabbit immunized with adult *A. viteae* homogenate. On contrary, *L. carinii* infected cotton rat sera and *B. malayi* infected mastomys sera did not reveal any cell-adherence property. Nevertheless, both these sheathed microfilariae exhibited cellular-adherence when immune rabbit sera were used in the test (77.5% *L. carinii*, 79.3% *B. malayi* mf). In case of *B. malayi*, serum of mouse immunised with *B. malayi* mf homogenate and from mastomys infected with *A. viteae* also induced some level of cell-adherence (48.6% and 56.7% respectively) (Table 1).

Normal mastomys, cotton rat, rabbit sera or medium containing guinea pig serum did not induce cell-adherence to mf *in vitro* in neither of the species.

In case of *A. viteae*-mastomys system neither complement nor heat-labile antibody (possibly IgE) plays any significant role in mediating adherence of splenocytes to mf *in vitro*. However, in case of sheathed mf of *L. carinii* and *B. malayi*, deployment of complement (by heating sera at 56°C for 30 min.) reduced the adherence capability of cells to homologous mf to a great degree. Thus % adhesion reaction which was 79.3, decreased to 28.6 after decomplementation of *B. malayi* rabbit serum and from 77.9% to 12.5% in case of *L. carinii* rabbit serum. Further heat treatment of immune rabbit sera at 60°C/2 hrs reduced the cell-adhesion reaction which was restored after the addition of fresh guinea pig serum as a source of complement (Table 1).

With the increasing dilutions of immune sera, % cell-adherence and intensity of cell-adherence decreased. Maximum cell-adhesion to mf occurred when undiluted serum was used. More than 1:128 dilution resulted into nominal or no cell-adherence to mf (Fig. 1).

Significant cell-adherence to blood mf of *A. viteae* was observed (maximum 91.5%) with 120 d old serum. Uterine mf had markedly lower (47.3%) cellular adherence (90–120 d serum). Percent cell-adherence started declining in presence of sera of mastomys harbouring 200 days old infection (Fig. 2).

Effect of antifilarials on *in vitro* cell-adherence to mf: (Fig. 3, 4)

All the piperazine derivatives showed significant enhancement of adherence capacity of splenocytes to all the three species of mf *in vitro* in presence of specific antibody. Maximum cell-adherence promoting capacity was exhibited by N-oxide of DEC followed by centperazine and DEC in that order. Levamisole did not exert similar cell-adhesion promoting ability to mf of all the three species, however, results in *B. malayi* with levamisole were erratic as in 2 experiments it killed most of the mf *in vitro* at higher concentration besides exerting cell-adherence in almost 100% mf. In rest of the experiments it did not modify the degree of cell-adherence. In case of *A. viteae*, levamisole had rather cell-adhesion suppressing action (2.2% decrease).
<table>
<thead>
<tr>
<th>Filarial sp.</th>
<th>Serum/Antibody</th>
<th>% Adherence of splenocytes</th>
<th>Splenocytes/mf</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. viteae</em></td>
<td>Medium only + guinea pig serum (G. pig se)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>NMS</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>NRS</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>IRS</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>IMS</td>
<td>91.5</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>IMS(DC)</td>
<td>75</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>IMS(60°C/1hr)</td>
<td>75.6</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>IMS</td>
<td>FR. I(19S)</td>
<td>85.9</td>
<td>++++</td>
</tr>
<tr>
<td>FR. II(7S)</td>
<td>6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>FR. I (without complement)</td>
<td>85.9</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>FR. I (Guinea pig serum)</td>
<td>90</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>B. malayi</td>
<td>Medium only + G. pig se</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>NMS</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>NRS</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>IMS</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Immunised mice sera(mf Ag)</td>
<td>48.6</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>IMS(A. viteae infected)+G. pig se</td>
<td>56.7</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>IRS(B. malayi adult Ag)</td>
<td>79.3</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>IRS( &quot; &quot; ) (DC)</td>
<td>28.6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IRS( &quot; &quot; ) 60°C/2hrs</td>
<td>22.2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IRS( &quot; &quot; ) 60°C/2hr + G. pig se</td>
<td>81.5</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>IRS</td>
<td>FR. I(19S)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>FR. II(7S)G. pig se</td>
<td>75.5</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>L. canni</td>
<td>Medium only + G. pig se</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>NCRS</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>NRS</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ICRS</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>IRS(Adult L. canni Ag.)</td>
<td>77.9</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>IRS(DC)</td>
<td>12.5</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IRS(60°C - 1hr)+ G. pig se</td>
<td>67.8</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>IRS</td>
<td>FR. I(19S)+ G. pig se</td>
<td>7.1</td>
<td>-</td>
</tr>
<tr>
<td>FR. II(7S)+ G. pig se</td>
<td>65.5</td>
<td>++++</td>
<td></td>
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- : No adherence
+ : 5–10 cells adhered/mf
++ : 10–25 cells adhered/mf
+++ : 25–75 cells adhered/mf
++++: >75 cells adhered/mf

NMS, Normal Mastomys Serum; NRS, Normal Rabbit Serum; IMS, Infected Mastomys Serum; IRS, Immune Rabbit Serum; NCRS, Normal Cotton Rat Serum; ICRS, Infected Cotton Rat Serum; DC, Docomplemented.
Fig. 1: Cell-adherence to blood and uterine microfilariae of *A. viteae* using undiluted serum of infected mastomys.

- Blood MF;  
- Uterine MF

+ 5–10 cells; ++ 10–25 cells/mf; +++ 25–75 cells; ++++ > 75 cells/mf

Fig. 2: Effect of sera dilution on cell-adherence.

- • *B. malayi*-immune rabbit serum; × × × *A. viteae*-infected mastomys serum; ○ ○ ○ *L. carinii*-immune rabbit serum
Fig. 3: *In vitro* cell-adherence to microfilariae in presence of microfilaricides.

- □ *A. viteae*; □ *B. malayi*; □ *L. carinii*; vertical bars

(↑) indicate ± SE and thick transverse bands.

(−) denote cell-adherence in untreated controls under identical condition.

Fig. 4: Changes in cell-adherence reaction to microfilariae by addition of microfilaricides *in vitro*.

- □ *B. malayi*; □ *A. viteae*; □ *L. carinii*
A. viteae required higher concentration of antifilarial agents for cell adhesion promotion action (1000–125 μg/ml) than B. malayi (500–15.7 μg/ml) or L. carinii (250–7.8 μg/ml).

DISCUSSION

The precise mode of action of DEC still remains unclear. Promotion of in vitro cellular adhesion reaction by exposing the antigenic determinant sites of microfilariae has been speculated in few recent years (Piessens and Beldekas, 1979; Chandrashekhar et al. 1984) for which specific antibody is essentially required with or without involving complement. Do other antifilarials act in the same way or is it an exclusive property of DEC or all piperazine compounds behave in the same manner as DEC regarding cell-adhesion promotion? These are the queries not yet solved. The disappearance of microfilaraemia in L. carinii infected albino rats is associated with adhesion of macrophages, lymphocytes and neutrophils to mf in the pleural cavity (Bagai and Subrahmanyam, 1970). In vitro adherence of cells to mf in presence of specific antibody is therefore regarded as an important phenomenon.

Four microfilaricides including three piperazines and a non piperazine compound have been used in the present study to explore their mechanism of action by modifying in vitro cellular adherence to mf. The findings suggested that adhesion promoting activity resides not only in DEC but is also possessed by other active piperazine analogues. Levamisole, although a strong microfilaricidal agent, does not affect the in-vitro cell-adherence phenomenon. However the results in B. malayi with this agent do not give a conclusive idea, as in 3 out of 5 experiments it did not affect cell-adhesion reaction while in rest two experiments it killed mf of B. malayi and also cell-adhesion to mf was almost 100%, which showed a slow decrease with decreasing concentration of drug. Sim et al. (1983) working with infective larvae (L₃) reported significant decrease in motility, greater cellular adherence and subsequent surface damage to larvae of B. malayi in presence of sera from levamisole treated patients. It was also observed in the present study that cells did not adhere or attack the dead or immotile microfilariae. They adhered well to the active mf only. Since undiluted immune serum caused very high degree of adherance, it was optimally diluted to yield around 40–50% adherence in order to evaluate the increase or decrease after the addition of antifilarials.

There was some difference in the adhesion promoting activity of different piperazines. All the species of mf (sheathed and unsheathed) were affected to the same degree by each agent. N-oxide, a major metabolite of DEC, enhances in vitro cellular adherence to mf to the maximum extent. Centperazine, another piperazine derivative exerted more potentiation of cell-adherence than DEC-the standard filaricide. Thus, it appears that all the piperazine analogs act on mf by promoting cell-adherence to them in a similar fashion as DEC. However, levamisole – a levorotatory isomer of tetramisole might be killing mf by different mechanism. Direct action of this agent on neuromuscular system and carbohydrate metabolism of mf is well reported (Subrahmanyam, 1987). We have
recently observed that cell-adherence to microfilariae can be modified by altering the surface using various surfactants (unpublished). It may be possible that piperazine compounds also alter the surface of microfilariae resulting into enhanced cellular adherence to them. Exposure of antigenic determinant sites on the surface of microfilariae by DEC makes them more vulnerable to immune attack (Hawking et al., 1950; Piessens and Beldekas, 1976).

Complement is essentially required in in vitro killing of sheathed mf of B. malayi and L. carinii. On contrary, in vitro cell-adherence to unsheathed mf of A. viteae occurs even in absence of complement. This condition remained unaltered even after the addition of antifilarials. Rao et al. (1987) observed ivermectin induced increased in vitro cell-adhesion to A. viteae mf in presence of IgM antibody involving complement using infected mastomys serum. The possible mechanism of mf killing by promoting cell-adherence in vitro is still unclear, however, there is a direct action of drugs on mf rather than cells as preincubation of mf with DEC increases such toxicity (Subrahmanyam, 1987).

It was interesting to observe that infected cotton rat (L. carinii) and mastomys (B. malayi) sera were unable to induce in vitro cell-adhesion phenomenon to homologous mf but strong adhesion occurred when rabbit sera hyper-immunised with homologous antigen was used. The reason could be the presence of IgG antibody which plays a major role in cell-adherence phenomenon in both species of sheathed mf (Table 1). It was revealed clearly when Fr. II of immune sera containing IgG (7 S) induced cell-adherence to B. malayi and L. carinii mf but not Fr I containing IgM (19 S). According to IgM-IgG shift immunisation of animals results into the development of IgM for a very short period followed by strong and persistent appearance of IgG (Uhr and Finkelstein, 1963). Formation of immune complexes may also be one of the factors responsible for failure of mastomys and cotton rat sera in inducing in vitro cell-adherence (Karavodin and Ash, 1982). However, disappearance of mf in A. viteae infected hamsters is associated with the appearance of anticuticular antibodies in infected sera (Weiss, 1978). In contrast to sheathed mf, the unsheathed mf of A. viteae required IgM antibody in cellular adhesion reaction. Tanner and Weiss (1978) also reported IgM induced in vitro cellular cytotoxicity to A. viteae mf in case of hamsters.

It is believed that mf which are present in the uteri of female worms and have not yet come in contact with the host tissue are different than those circulating in the blood (Wegerhof and Wenk, 1979). Blood mf of various species of filariae incorporate host tissue factors on their surfaces (Philipp et al., 1984; Maizels et al., 1984). Cell-adherence assay was, therefore, performed with these two populations of mf of A. viteae and very little cell-adhesion occurred to uterine mf as compared to those derived from blood. It is not clear, why uterine mf which are more vulnerable to host’s immune attack did not exhibit intense adherence of cells onto their surface. Maximum cell-adhesion occurred around day 120 post-infection, which may be attributed to the high level of IgM antibody response during this time (Weiss, 1978; Singh et al., 1988).

It can therefore be concluded that all the piperazine agents used in the study act on mf in the same way by promoting in vitro adhesion of cells to mf (both sheathed and unsheathed). However a nonpiperazine agent levamisole does not possess this property. In
unsheathed *A. viteae* mf the reaction is complement independent and utilises IgM while in sheathed mf of *B. malayi* and *L. carinii* adhesion occurs in presence of complement and IgG.

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